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Therapeutics, Targets, and Chemical Biology

Cancer Research

Characterization of a c-Rel Inhibitor That Mediates Anticancer Properties in Hematologic Malignancies by Blocking NF-κB–Controlled Oxidative Stress Responses

Yusuke Shono[1], Andrea Z. Tuckett[1], Hsiou-Chi Liou[2], Ekaterina Doubrovina[3], Enrico Derenzini[4], Samedy Ouk[2], Jennifer J. Tsai[1], Odette M. Smith[1], Emily R. Levy[1], Fabiana M. Kreines[1], Carly G.K. Ziegler[1,5], Mary I. Scallion[4], Mikhail Doubrovin[3], Glenn Heller[6], Anas Younes[4], Richard J. O'Reilly[3], Marcel R.M. van den Brink[1,4], and Johannes L. Zakrzewski[3]

Abstract

NF-κB plays a variety of roles in oncogenesis and immunity that may be beneficial for therapeutic targeting, but strategies to selectively inhibit NF-κB to exert antitumor activity have been elusive. Here, we describe IT-901, a bioactive naphthalenethiobarbiturate derivative that potently inhibits the NF-κB subunit c-Rel. IT-901 suppressed graft-versus-host disease while preserving graft-versus-lymphoma activity during allogeneic transplantation. Further preclinical assessment of IT-901 for the treatment of human B-cell lymphoma revealed antitumor properties *in vitro* and *in vivo* without restriction to NF-κB–dependent lymphoma. This nondiscriminatory, antilymphoma effect was attributed to modulation of the redox homeostasis in lymphoma cells resulting in oxidative stress. Moreover, NF-κB inhibition by IT-901 resulted in reduced stimulation of the oxidative stress response gene heme oxygenase-1, and we demonstrated that NF-κB inhibition exacerbated oxidative stress induction to inhibit growth of lymphoma cells. Notably, IT-901 did not elicit increased levels of reactive oxygen species in normal leukocytes, illustrating its cancer selective properties. Taken together, our results provide mechanistic insight and preclinical proof of concept for IT-901 as a novel therapeutic agent to treat human lymphoid tumors and ameliorate graft-versus-host disease. *Cancer Res; 76(2); 377–89. ©2016 AACR.*

Introduction

Amongst its many functions, NF-κB plays important roles in immunity (1–6) and oncogenesis (1, 7), indicating that therapeutic targeting of this pathway could be beneficial in a variety of clinical settings; however, an NF-κB–specific inhibitor does not exist in clinical practice to date. One approach toward development of such a compound is small-molecule–mediated direct inhibition of one or several members of the NF-κB family of transcription factors, a network that comprises five structurally related proteins including p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB, and c-Rel (8).

After screening of a library of 15,000 small molecules with a biochemical assay measuring inhibition of c-Rel interaction with its high-affinity DNA-binding site, we identified two scaffolds with inhibitory activity for the NF-κB family of transcription factors and particularly high specificity for the transcription factor c-Rel. These scaffolds, thiohydantoin and napthalenethiobarbitutate, act as direct NF-κB inhibitors by preventing DNA binding of the c-Rel protein. We previously reported that *in vitro* treatment of T cells with the thiohydantoin IT-603 induces c-Rel deficiency, resulting in suppression of T-cell alloactivation without compromising T-cell activation triggered by recognition of tumor-associated or viral antigens (9). Here, we demonstrate significant *in vivo* efficacy of the naphthalenethiobarbiturate IT-901 in mouse models of graft-versus-host disease (GVHD) and graft-versus-lymphoma (GVL), as well as a xenograft model of human B-cell lymphoma, revealing that IT-901 treatment results not only in suppression of GVHD while retaining GVL activity, but it also mediates promising antilymphoma effects. We characterize pharmacokinetic (PK) and toxicology profiles as well as the mechanism of action of IT-901–mediated antilymphoma activity in human diffuse large B-cell lymphoma (DLBCL) cells, thereby laying the groundwork for the development of a new drug combining unique immunomodulatory and antineoplastic properties.

[1]Department of Immunology, Memorial Sloan Kettering Cancer Center, New York, New York. [2]ImmuneTarget Inc., San Diego, California. [3]Department of Pediatrics, Memorial Sloan Kettering Cancer Center, New York, New York. [4]Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, New York. [5]Department of Computational Biology, Memorial Sloan Kettering Cancer Center, New York, New York. [6]Department of Epidemiology and Biostatistics, Memorial Sloan Kettering Cancer Center, New York, New York.

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M.R.M. van den Brink and J.L. Zakrzewski contributed equally to this article.

Corresponding Author: Johannes L Zakrzewski, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY 10065. Phone: 212-639-3267; Fax: 212-717-3447; E-mail: ZakrzewJ@mskcc.org

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Materials and Methods

Mice and bone marrow transplantation

We obtained female C57BL/6 (B6, H-2^b), BALB/c (H-2^d) from the Jackson Laboratory. Male *NOD/scid/IL2Rγ*(null; NSG) mice were purchased from the Jackson Laboratory. BALB/C-Tg(NFkB-RE-luc)-Xen mice were obtained from Taconic. B6 mice carrying the *c-Rel* gene null mutation (*c-Rel*$^{-/-}$) were originally generated by inserting the neomycin cassette into the fifth exon of the c-Rel gene (3). *c-Rel*$^{-/-}$ B6 mice were maintained at Memorial Sloan Kettering Cancer Center (New York, NY) in accordance with Institutional Animal Care and Use Committee Standards. Mice used for experiments were 6 to 9 weeks old. Mouse HSCT experiments were performed as previously described (10), with 850 cGy split-dosed lethal irradiation of BALB/c recipients transplanted with bone marrow (5×10^6), T cell depleted (TCD) with anti-Thy-1.2 and low-TOX-M rabbit complement (Cedarlane Laboratories). Mouse T cells were prepared by harvesting donor splenocytes and enriching T cells by Miltenyi MACS purification of CD5 (routinely >90% purity). In A20 lymphoma experiments, animals received tumor cells intravenously in a separate injection on day 0.

Small-molecule c-Rel inhibitor compounds

Our team previously identified thiohydantoin and naphthalenethiobarbiturate derivatives as conformation-disrupting direct c-Rel inhibitors (9, 11, 12). c-Rel inhibitory activity of these small-molecule compounds was confirmed by fluorescence polarization (FP) as well as electrophoretic mobility shift assay (EMSA) utilizing the DNA-binding property of the c-Rel protein. NF-κB DNA-binding ELISA (TransAM NFκB Family kit from Active Motif North America, see the manufacturer's protocol for more details) was also used to evaluate the activation of c-Rel. To obtain an estimate of the IC_{50}, a constant amount of 32p-labeled-κB probe (1 nmol/L, CD28RE) and c-Rel protein (5 nmol/L) was incubated with serial dilutions of IT-901 (50 μmol/L to 100 nmol/L) for 20 minutes in a 20-μL reaction at room temperature. The reactions were then resolved on native polyacrylamide gel following the standard protocol described previously (13). The c-Rel inhibitors IT-603 and IT-901 were provided by ImmuneTarget Inc.

Assessment of GVHD and GVT; *in vivo* BLI

Mice were monitored daily for survival and weekly for GVHD clinical scores (14). In GVT experiments, we determined the bioluminescent signal intensity (BLI) of tumor-bearing mice twice weekly as described previously (15). We superimposed pseudocolor images showing the whole-body distribution of bioluminescent signal intensity on grayscale photographs and determined total flux (photons/s) for individual mice. We determined the cause of death (tumor vs. GVHD) by necropsy and histopathology as previously described (16).

Cell lines and primary cells

A20, a B-cell lymphoma cell line of BALB/c origin, was kindly provided by A. Houghton (Memorial Sloan Kettering Cancer Center). We retrovirally transduced A20 to express a triple fusion protein consisting of herpes simplex virus thymidine kinase, enhanced GFP, and firefly luciferase (TGL) as described previously (17). The human DLBCL-derived cell lines SU-DHL4, OCI-Ly19, U2932 were obtained from the German Collection of Microorganisms and Cell Cultures, Department of Human and Animal Cell Cultures (Braunschweig, Germany); SU-DHL8 cells, were obtained from ATCC. The HBL1 and TMD8 cells were kindly provided by Dr. R.E. Davis (Houston, TX), and authenticated before using them by the MD Anderson Characterized Cell Lines Core Facility. Cell lines were cultured in RPMI-1640 medium supplemented with 10% to 20% heat-inactivated FBS (GIBCO BRL), 1% L-glutamine, and penicillin-streptomycin in a humid environment of 5% CO_2 at 37°C. An NF-κB/Jurkat/GFP transcriptional reporter cell line (18) was purchased from System Biosciences. Cells were stimulated with human TNFα (10 ng/mL) and incubated in the presence of IT-603, IT-901, or empty vehicle for 20 hours. NF-κB activity (GFP fluorescence intensity) and viability (based on DAPI) were analyzed by flow cytometry. Combined data from three independent experiments are presented. Human $CD19^+$ peripheral blood mononuclear cells (PBMC) were purchased from United States Biological. Human primary B-cell lymphoma and T-cell lymphoma cells were obtained from the Hematology/Oncology Tissue Bank at Memorial Sloan Kettering Cancer Center. Sample and patient data collection were approved by the Human Biospecimen Utilization Committee and the Institutional Review Board/Privacy Board-B of Memorial Sloan Kettering Cancer Center.

Human EBV-transformed B lymphoblastoid cells were generated from PBMC of a healthy donor. Human EBV-specific T cells were generated from PBMC of the same donor by repeated *in vitro* stimulations with an autologous EBV-transformed B cells.

Serum cytokines analyses

Blood was collected into microcentrifuge tubes, allowed to clot and centrifuged, and the supernatant was collected. Multiplex ELISA was conducted per manufacturer's instructions (Millipore). Results were acquired with a Luminex 200 instrument and analyzed with xPONENT software (Luminex Corporation).

Antibodies and flow cytometry

All antibodies other than the anti–c-Rel, anti-p50, anti-p52 antibodies (Santa Cruz Biotechnology), and the anti-CD44, anti-p65 antibodies (Biolegend) were obtained from BD Biosciences – Pharmingen. For cell analysis of surface markers, cells were stained for 20 minutes at 4°C in PBS with 0.5% BSA (PBS/BSA) after Fc block, washed, and resuspended in DAPI in PBS/BSA. Cell surface staining was followed by intracellular staining with the eBioscience kit as per the manufacturer's instructions. Dead cells were excluded with LIVE/DEAD Fixable Dead Cell Stain kit (Invitrogen). Intranuclear c-Rel staining was performed as previously described (9). All flow cytometry was performed on an LSR II (BD Biosciences) and analyzed with FlowJo (TreeStar Software). Human anti-adalimumab was purchased from AbD Serotec (Bio-Rad Laboratories, Inc).

MTS assay

Relative cell growth was measured by the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega) as per the manufacturer's instructions. Briefly, tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] was added to the culture media and the conversion of MTS into formazon was measured by the amount of 490 nm absorbance. The number of viable cells correlates with absorbance at 490 nm (see the manufacturer's protocol for more details).

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qPCR

Reverse transcription-PCR was performed with iScript Advanced cDNA Synthesis Kit for RT-qPCR (BIO-RAD). For real-time PCR, NF-κB signaling pathway Plus PrimePCR Pathway Plate was used per manufacturer's instructions (BIO-RAD). PCR was done on CFX96 Touch Real-Time PCR (Bio-Rad) with Universal SYBR Green Supermix (BIO-RAD). Relative amount of HMOX1 was calculated by the comparative ΔC(t) method utilizing CFX Manager Software (BIO-RAD).

Heme oxygenase-1 ELISA

In order to measure heme oxygenase-1 (HMOX1) protein level in IT-901–treated Ly19 human DLBCL, Ly19 cells were lysed with extraction reagent included in the HO-1 Human ELISA Kit (Abcam). Analysis was performed per manufacturer's instructions. Optical density (OD) was determined, using a microplate autoreader (Bio-Tek Optiplex 755 Microplate Reader) at 450 nm. HMOX1 protein concentration of the samples was determined based on a specific HMOX1 standard curve.

Mouse IL2 ELISA

IL2 production by activated T cells was measured with the Mouse IL-2 ELISA Kit purchased from Sigma-Aldrich. Wild-type C57BL/6 mouse splenocytes were treated with different concentrations of IT-901 or empty vehicle for one hour and stimulated *in vitro* with anti-CD3/CD28 for 5 hours. Cell culture supernatants were analyzed for IL2 as per the manufacturer's instructions.

Inducible nitric oxide synthase ELISA

An ELISA-based assay was performed to measure total inducible nitric oxide synthase (iNOS) in IT-901–treated human DLBCL including Ly19, SU-DHL8, and TMD8 cells. Cells were treated *in vitro* with IT-901 (4 μmol/L) or control solution for 24 hours in a 96-well microplate. After fixation of cells in the wells, iNOS levels were determined using Human Total iNOS Cell-Based ELISA (R&D Systems) as per the manufacturer's instructions.

Detection of reactive oxygen species and treatment with *N*-Acetyl-L-cysteine

B-cell lymphoma cells were treated *in vitro* with IT-901 (4 μmol/L) or control solution for different time periods to induce reactive oxygen species (ROS) generation. ROS levels were analyzed by utilizing the Image-iT LIVE Green Reactive Oxygen Species Detection Kit (Molecular Probes, Invitrogen) as per the manufacturer's instructions. ROS levels were analyzed by quantifying a fluorogenic marker (carboxy-H_2DCFDA) using flow cytometry (LSR II, BD Biosciences). *N*-Acetyl-L-cysteine was purchased from SIGMA-ALDRICH. For microscopic detection of ROS, IT-901–treated cells were stained with carboxy-H_2DCFDA, MitoTracker Deep Red FM (Life Technologies), and Hoechst 33342 (Life Technologies), followed by laser scanning confocal microscopy on a Leica SP5 confocal microscope.

Detection of reactive oxygen and reactive nitrogen species

Analyses of superoxide, hydrogen peroxide, and reactive nitrogen levels were performed with IT-901–treated human DLBCL including Ly19, SU-DHL8, and TMD8 cells. Cells were treated *in vitro* with IT-901 (4 μmol/L) or control solution for 24 hours and the levels of superoxide, hydrogen peroxide and reactive nitrogen were determined by MitoSOX Red Mitochondrial Superoxide Indicator, Premo Cellular Hydrogen Peroxide Sensor, and DAF-FM Diacetate, respectively, as per the manufacturer's instructions (Life Technologies). The fluorescence intensities of cells after treatment were detected by flow cytometry (LSR II, BD Biosciences).

Glutathione assay

Ly19, SUDH8, and TMD8 cells were treated with IT-901 at 4 μmol/L or control vehicle for 24 hours and cells were analyzed for total and oxidized glutathione by the HT Glutathione Assay Kit (Trevigen Inc.) as per the manufacturer's protocol. Reduced GSH was calculated by subtracting oxidized GSSG from total glutathione.

Mitochondrial respiration assay

Real-time measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in Ly19 cells were conducted in a XF96 extracellular flux analyzer (Seahorse Bioscience). Briefly, Ly19 cells treated either with IT-901 at 4 μmol/L or control vehicle 3 to 24 hours were washed with RPMI-1640 supplemented with 10% FBS and L-glutamine, resuspended in mitochondria stress medium (DMEM supplemented with 10 mmol/L glucose, 1 mmol/L pyruvate, 2 mmol/L glutamine, pH 7.35) and transferred to a XF96 cell culture microplates (Seahorse Bioscience) pretreated with Cell-Tak (BD Biosciences) at a density of 200,000 cells per well. After incubation at 37°C in the absence of CO2 for 1 hour in a Prep Station incubator Station, real-time measurements of OCR were collected at intervals of 4 minutes as a parameter of mitochondrial respiration. The standard assay was carried out and used the compounds stock provided in the XF Cell Mito Stress Test Kit. After optimization of oligomycin and FCCP working concentration, the mitochondrial stress assay was conducted following the manufacturer's instructions.

c-Rel knockdown with shRNA

An optimized miRNA backbone (miR-E) for effective c-Rel knockdown was designed according to a previous report (19). We designed 12 different sequences for efficient c-Rel targeting by shRNA and utilized two (#2131 and #2205) in our experiments to analyze the HMOX1 level in c-Rel knocked-down Ly19 lymphoma cells. 97-mir sequences of the two shRNAs are TGCTGTTGACAGTGAGCGACAGCATTTTGTATTTGTCTAATAGTGAAGCCACAGATGTATTAGACAAATACAAAATGCTGCTGCCTACTGCCTCGGA (#2131) and TGCTGTTGACAGTGAGCGCAATACTGTATTTGAGAATATATAGTGAAGCCACAGATGTATATATTCTCAAATACAGTATTATGCCTACTGCCTCGGA (#2205). Ly19 cells were resuspended in lentivirus-containing supernatant in the presence of polybrene (4 ng/μmL) and incubated at 37°C in a CO_2 incubator for 24 hours. Transduced cell populations were selected 7 days after infection using 0.5 ng/μL puromycin (Sigma-Aldrich).

Analysis of c-Rel gene expression in human cells

c-Rel expression data from human primary cells and cell lines were obtained from several publically available databases. Microarray data normalized by RMA and RNA sequencing data normalized by RPKM were obtained from The Broad Institute of MIT and Harvard (20, 21).

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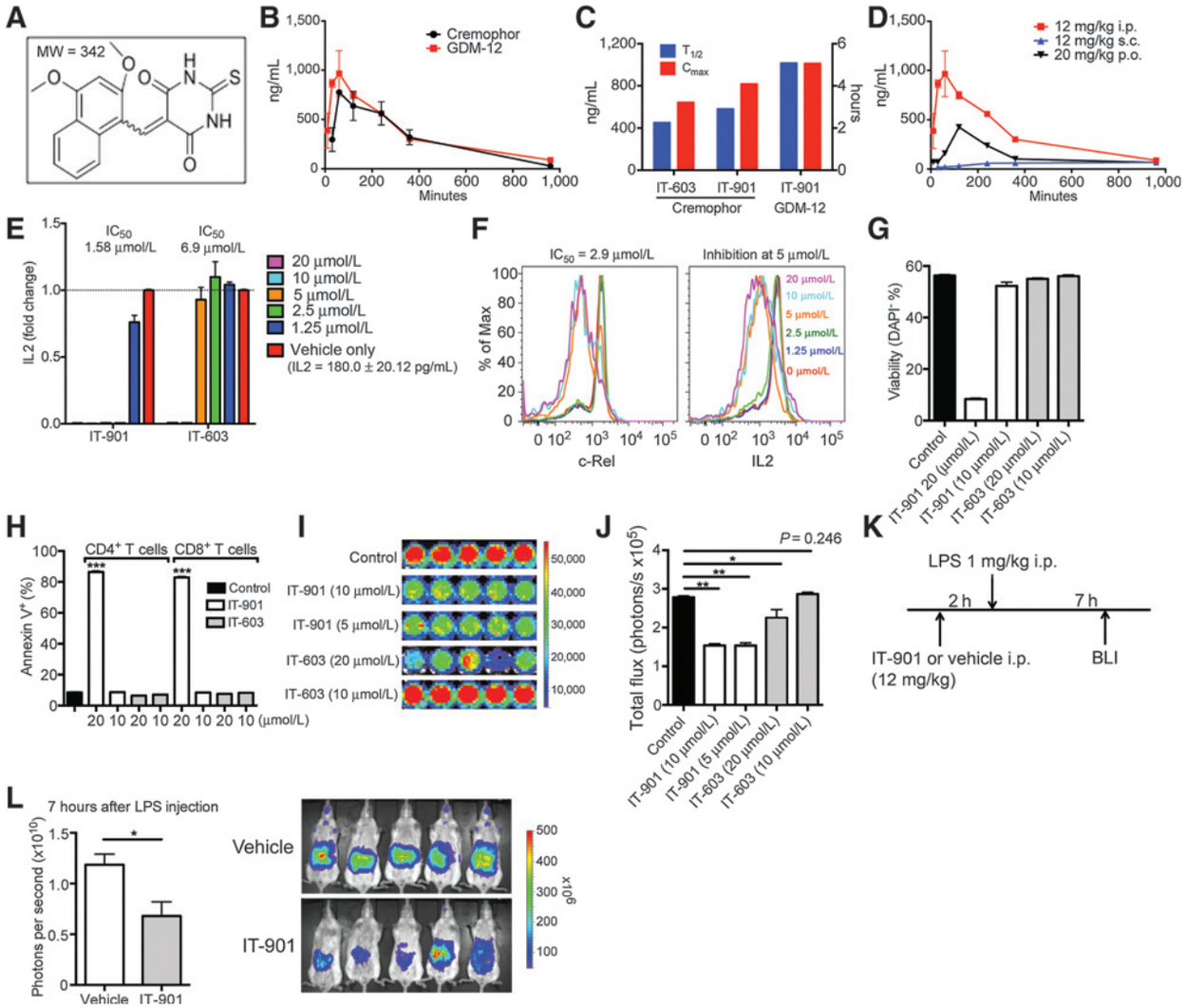

Figure 1.

IT-901 is a more potent c-Rel inhibitor than IT-603 and has a superior pharmacokinetic profile. A, structure of IT-901. B–D, plasma samples were analyzed at 30 minutes, 1, 2, 4, 6, and 16 hours after intraperitoneal administration of c-Rel inhibitor compounds IT-603 and IT-901. To assess the levels of IT-603 and IT-901 in blood, samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS; for more details, see Materials and Methods). B, pharmacokinetics of IT-901 utilizing different vehicles are shown. C, serum half-life ($T_{1/2}$) and maximum concentration (C_{max}) of IT-603 and IT-901 with different vehicles are presented. D, pharmacokinetics of IT-901 with GDM-12 with different types of administrations are shown. i.p., intraperitoneal injection; s.c., subcutaneous injection; p.o., per oral. E and F, wild-type C57BL/6 mouse splenocytes were treated with six different concentrations of IT-901 for 1 hour and analyzed after anti-CD3/CD28 stimulation for 5 hours. E, levels of IL2 evaluated by ELISA in the culture supernatant are shown. F, representative flow-cytometric analysis of intracellular c-Rel and IL2 are shown. Data are representative of more than three experiments. G and H, viability of the T cells after 24-hour incubation with different concentrations of IT-603, IT-901, or empty vehicle. Percentages of live/dead cells and Annexin-V–positive proapoptotic cells are shown. Values represent mean ± SEM ($n = 5$, technical replicates). ***, $P < 0.001$ comparing IT-901 to empty vehicle. Data are representative of more than three experiments. I and J, BALB/C-Tg(NFκB-RE-luc)-Xen mice were used for *in vitro* and *in vivo* analyses of c-Rel inhibitor compounds' effects on NFκB signaling. A total of 1×10^6 splenocytes from *NFκB-RE-luc* mice were prepared *in vitro* with 1 μg/mL of LPS on a 96-well plate. Cells were treated with different concentrations of IT-901, IT-603, or empty vehicle (control) for 24 hours and bioluminescent intensity was analyzed. I, pseudocolor images superimposed on conventional photographs are shown. J, the bioluminescent signal intensity is shown. Data are representative of three independent experiments (I and J). Values represent mean ± SEM ($n = 3$, technical replicates). *, $P < 0.05$; **, $P < 0.01$. K, *in vivo* treatment schedule of *NFκB-RE-luc* mice. L, the whole-body activation of NF-κB signal was monitored using *in vivo* bioluminescence imaging (BLI). Pseudocolor images superimposed on conventional photographs are shown. Data are representative of two independent experiments (K and L). Values represent mean ± SEM ($n = 5$); *, $P < 0.05$.

Pharmacokinetics

Plasma samples were analyzed at 30 minutes, 1, 2, 4, 6, and 16 hours after intraperitoneal, subcutaneous, or oral administration of 12 to 20 mg/kg of c-Rel inhibitor compound IT-603 and IT-901. To assess the level of IT-603 and IT-901 in blood, samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described (22, 23). Calibration curves were determined for IT-603 to permit conversion of peak areas to the drug amounts against external reference standards. The tandem MS/MS detector (Model ABI/Sciex API 4000, Applied

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Biosystem) permitted verification of peak identity as well as a quantitative assessment of the compounds in the samples.

Xenograft model of EBV-induced B-cell lymphoma

Male NSG mice received 5×10^6 EBV-transformed B lymphoblastoid cells that were transduced to express firefly luciferase via subcutaneous injection on the back left side above the hind leg. On day 8 after tumor challenge, animals were assigned to the different groups and treatment with IT-901, vehicle, and/or EBV-specific human T cells was initiated. A total of 2,000 units of human IL2 were administered i.p. to all animals three times per week starting on day 8 after tumor challenge. Tumor progression was monitored by *in vivo* BLI and by caliper measurement. Animals were sacrificed when tumors became ulcerated.

Statistical analysis

Data are presented as mean ± SEM. Survival curves were analyzed with the Mantel–Cox log-rank test. For longitudinal *in vivo* studies measuring tumor volume, mice were randomly assigned to the treatment groups and the area under the curve (AUC) was used to summarize the trajectory of the measured value of each mouse under study. Not all the mice were followed for the full length of the study. The primary reason for censoring was death or sacrifice, and ignoring this type of informative censoring may result in a biased treatment comparison. To eliminate this bias, a test statistic was used that is formed based on the information up to the minimum follow-up time for each cross treatment mouse pair. By eliminating the uneven censorship between mouse pairs in different groups, a test statistic can be constructed that has mean zero when the growth rates in the two groups are equal. The statistic is based on the average difference in the censored AUC curves between treatment groups. *P* values were generated from a permutation test, using the AUC and log rank test statistics. The application of the permutation procedure was due to the "small" number of animals in these studies. For nonsurvival pointwise analyses, unpaired *t* test was used for comparisons between two experimental groups, or nonparametric Mann–Whitney *U* test was used for non-Gaussian distributions, and ANOVA was used for comparisons of more than two groups. A *P* value of less than 0.05 was considered statistically significant.

Results

The c-Rel inhibitor IT-901 is a promising small molecule for drug development

We previously introduced the thiohydantoin IT-603 as a small-molecule c-Rel inhibitor, indicating that this intranuclear target is indeed druggable (9). After additional testing and structure activity relationship analysis we identified the naphthalenethiobarbiturate IT-901 (Fig. 1A) as a second chemical lead. Electrophoretic mobility shift assay (EMSA) and NF-κB DNA-binding ELISA revealed that IT-901 efficiently inhibits DNA binding of c-Rel (Supplementary Fig. S1A and S1B). The estimated half maximal inhibitory concentration (IC_{50}) regarding global NF-κB activity was about six times lower for IT-901 (3 μmol/L) compared with IT-603 (18.8 μmol/L), and compared with several reference drugs NF-κB inhibition by IT-901 was only outperformed by the proteasome inhibitor bortezomib (Supplementary Fig. S2).

Both IT-603 and IT-901 are hydrophobic but can easily be dissolved in DMSO. We previously reported that IT-603/DMSO was much less toxic than IT-901/DMSO when incubating T cells for 24 hours and we therefore initially used IT-603/DMSO treatment as our standard protocol for chemical induction of c-Rel

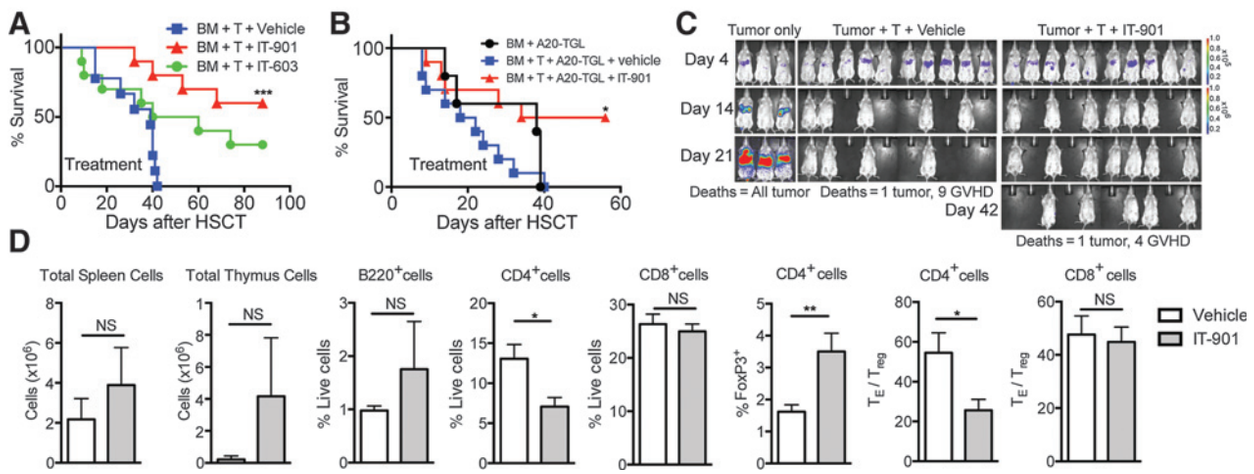

Figure 2.

c-Rel inhibitor administration is an effective treatment of acute GVHD without impairing antitumor activity. A, lethally irradiated BALB/c recipients were transplanted with C57BL/6 TCD BM cells with 0.5×10^6 C57BL/6 wild-type T cells. c-Rel inhibitor compound IT-603, IT-901, or control vehicle solution was administered i.p. from day 8 (24 mg/kg, every other day for 2 weeks). Survival curve is shown. ***, $P < 0.001$ comparing recipients treated with IT-901 versus vehicle. Data are representative of two independent experiments ($n = 10$). B and C, lethally irradiated BALB/c recipients were transplanted with C57BL/6 TCD BM cells with 0.5×10^6 C57BL/6 wild-type T cells. IT-901 or control vehicle solution was administered i.p. from day 8 (24 mg/kg, every other day for 2 weeks). On day 0, HSCT recipients were challenged with 0.25×10^6 luciferase-expressing A20-TGL B-cell lymphoma cells. Survival curve is shown (B). *, $P < 0.05$ comparing recipients treated with IT-901 versus vehicle. Data are representative of two independent experiments ($n = 3$–10). The whole-body distribution of tumor cells was monitored using *in vivo* BLI. For four time points, pseudocolor images superimposed on conventional photographs are shown (C). D, lethally irradiated BALB/c recipients were transplanted with C57BL/6 TCD BM cells with 0.5×10^6 C57BL/6 wild-type T cells. IT-901 or control vehicle solution was administered i.p. from day 8 (24 mg/kg, every other day for 2 weeks). Spleen and thymus were analyzed on day 21 after HSCT. Values represent mean ± SEM ($n = 5$). Data are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; NS, not significant.

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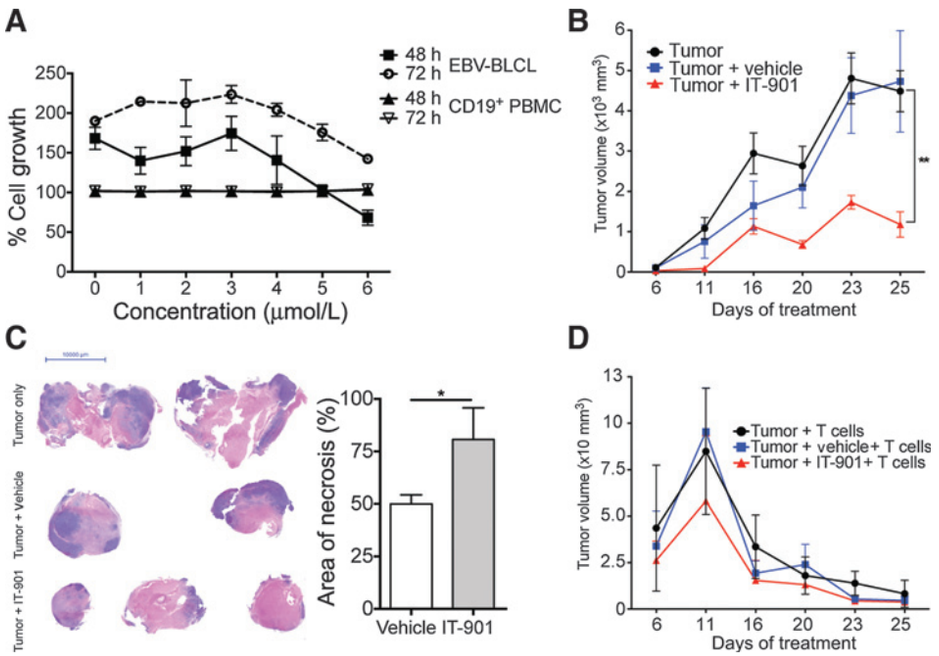

Figure 3.

IT-901 inhibits tumor growth in a xenograft model of human EBV-induced B-cell lymphoma. A, human EBV-BLCL and control human CD19$^+$ PBMC were cultured for 3 days in the presence of serial dilutions of IT-901. Relative cell growth was measured by MTS Cell Proliferation Assay. Mean ± SEM of technical replicates are presented. B–D, *in vivo* efficacy of IT-901 in a xenograft model of EBV-induced B-cell lymphoma. B, 5×10^6 EBV-BLCL were administered subcutaneously above the left hind leg of NSG mice. On day 8 after tumor challenge, treatment with IT-901 (20 mg/kg, i.p. daily) versus vehicle or no treatment was initiated. Tumor volume was measured manually with a caliper at the indicated time points. Mean ± SEM are presented ($n = 5$). One of two independent experiments is presented. **, $P < 0.01$. C, H&E staining of whole tumors harvested at day 25 after start of treatment. Left, representative images of tumors harvested from the three groups. Purple areas represent viable lymphoma cells, pink areas represent necrosis. Right, area of necrosis of whole tumors was determined based on H&E staining. Mean ± SEM are presented. *, $P < 0.05$. D, 5×10^6 EBV-BLCL were administered subcutaneously above the left leg of NSG mice. All animals received three infusions of 10×10^6 EBV-specific human cytotoxic T lymphocytes in weekly intervals starting day 8 after tumor challenge. In addition, treatment with IT-901 (20 mg/kg, i.p. daily) versus vehicle or no treatment was initiated on day 8 after tumor challenge. Tumor volume was measured manually with a caliper at the indicated time points. Mean ± SEM are presented ($n = 5$). One of two independent experiments is presented.

deficiency *in vitro* (9). In order to establish protocols for systemic administration of a c-Rel inhibitor drug in preclinical models, we formulated IT-603 and IT-901 with the FDA-approved nonionic surfactant Cremophor as the vehicle for PK studies. We found that intraperitoneally (i.p.) injected IT-901 had a superior PK profile compared with IT-603 (Supplementary Fig. S3). Utilizing the PEGylated synthetic lipid 1,2-dimyristoyl-rac-glycerol-3-dodecaethylene glycol (GDM-12; ref. 24) for compound formulation we further improved the PK profile of IT-901 (Fig. 1B) by increasing the half-life ($T_{1/2}$) and peak-concentration (C_{max}; Fig. 1C). Of note, both oral (p.o.) and especially subcutaneous (s.c.) administration of IT-901 resulted in much lower serum levels compared with i.p. administration (Fig. 1D).

We next compared the *in vitro* efficacy of IT-901/GDM-12 versus IT-603/GDM-12 in a T-cell–based assay (please note that the IC_{50} identified in these experiments will be different from the ones obtained by experiments utilizing DMSO as vehicle and/or biochemical instead of cell-based assays). IL2 is a well-known c-Rel target gene in activated T cells (2, 3, 9). We therefore used both flow cytometry and ELISA to measure IL2 expression in activated T cells to assess the efficacy of our c-Rel inhibitor compounds dissolved in 3% GDM-12. As expected, we found that IT-901 was again more potent than IT-603 (Fig. 1E and Supplementary Fig. S3). The IC_{50} of IT-901/GDM-12 was 2.9 μmol/L for c-Rel, whereas IL2 secretion was successfully blocked at 5 μmol/L (Fig. 1F). Importantly, in contrast with our previous observations with DMSO as the vehicle, cell viability was not compromised following 24 hours of incubation in the presence of IT-901/GDM-12 at concentrations up to 10 μmol/L (Fig. 1G and H). However, we noticed that concentrations above 10 μmol/L become increasingly toxic and may lead to apoptosis of healthy cells.

In order to confirm the inhibitory effect of IT-901 on NF-κB pathway activity both *in vitro* and *in vivo*, we utilized BALB/c-Tg (NFκB-RE-luc)-Xen mice that carry a transgene containing NF-κB–responsive elements and modified firefly luciferase cDNA (25). Incubation of LPS-stimulated splenocytes from *NFκB-RE-luc* mice in the presence or absence of c-Rel inhibitors demonstrated inhibition of NF-κB activity by both IT-603 and IT-901, again showing stronger inhibition by IT-901 (Fig. 1I and J). Moreover, *NFκB-RE-luc* mice injected with LPS following a single dose of IT-901 (Fig. 1K) showed significant inhibition of NF-κB activity seven hours after treatment (Fig. 1L), confirming that IT-901 inhibits c-Rel/NF-κB both *in vitro* and *in vivo*.

c-Rel inhibitor administration is an effective treatment of acute GVHD without impairing antitumor activity

Next, we performed toxicology studies demonstrating that healthy C57BL/6 mice treated with i.p. or high-dose p.o.

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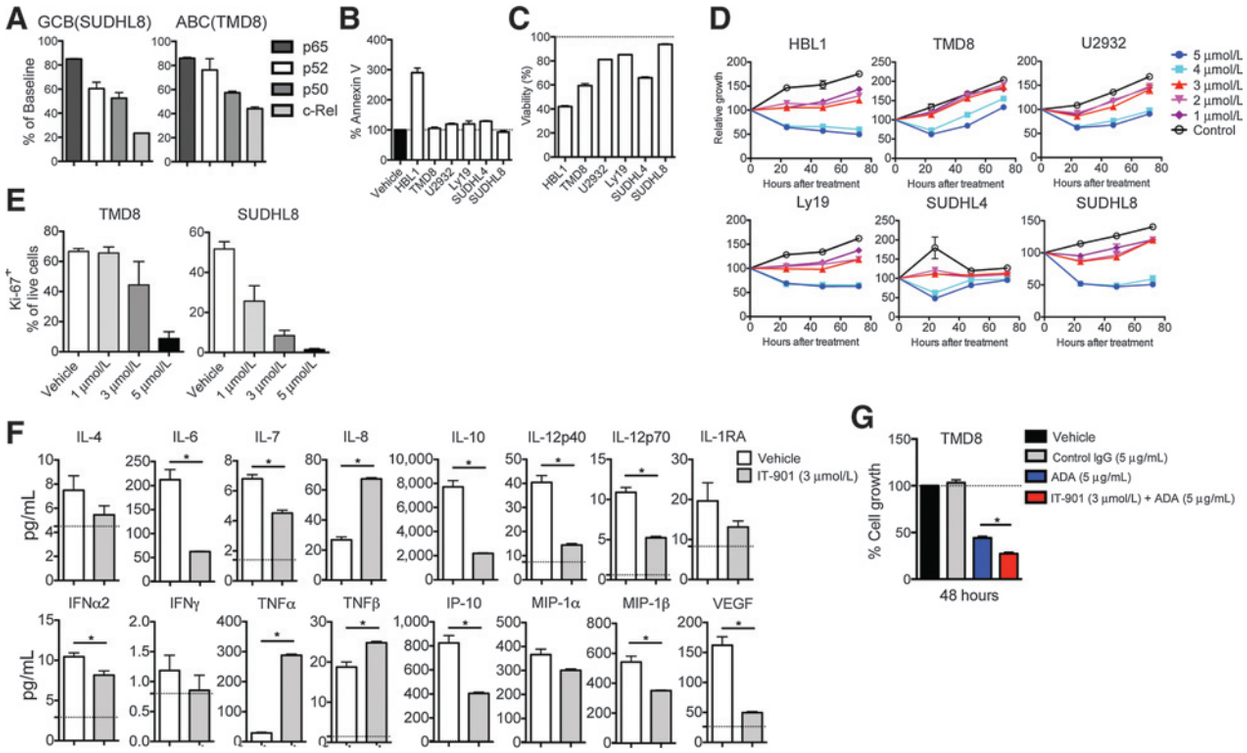

Figure 4.

IT-901 inhibits growth of human DLBCL and modulates the cytokine profile of activated B-cell lymphoma cells. A, SU-DHL8 and TMD8 cells were cultured in the presence of IT-901 (3 μmol/L) or control vehicle solution for 24 hours. Nuclear translocation of four NF-κB subunits (p65, p52, p50, and c-Rel) was analyzed by flow cytometry. Values represent mean ± SEM ($n = 2$, technical replicates). B and C, human DLBCL cell lines were treated with IT-901 (4 μmol/L) or control vehicle for 48 hours. Annexin-V–positive proapoptotic cells are shown (B). Viability of the tumor cells after 48-hour incubation is shown in C (normalized values to vehicle treatment). Values represent mean ± SEM ($n = 4$, technical replicates). Data are representative of two independent experiments. D, human DLBCL cell lines were treated for 3 days with serial dilutions of IT-901. Relative cell growth was measured by MTS Cell Proliferation Assay. Data are representative of three independent experiments. E, TMD8 and SU-DHL8 cells were cultured in the presence of IT-901 (1–5 μmol/L) or control vehicle solution for 24 hours and viability as well as Ki-67 expression was analyzed by flow cytometry. Percentages of Ki-67$^+$ proliferating viable cells are shown. Data are representative of two independent experiments. F, TMD8 tumor cells were cultured in the presence of IT-901 (3 μmol/L) or control vehicle solution for 24 hours. Cytokine levels of the supernatant are shown (Multiplex ELISA). Mean values and SEM are presented (samples were analyzed in triplicates). *, $P < 0.05$. Dotted line indicates the minimum detection limit of the assay. G, TMD8 tumor cells were cultured in the presence of IT-901 (3 μmol/L) and anti-TNFα antibody (ADALIMUMAB; ADA) for 48 hours. Relative cell growth was measured by MTS Cell Proliferation Assay. Values represent mean ± SEM ($n = 4$, technical replicates). *, $P < 0.05$. Data are representative of two independent experiments.

(150 mg/kg) IT-901 for 14 days had no clinical symptoms and no changes in their numbers of splenocytes, thymocytes, and T-cell subsets (Supplementary Fig. S5 and Supplementary Table S1). However, when administering IT-901 daily i.p. for more than 2 weeks we observed diarrhea, ruffled fur, and up to 10% weight loss.

On the basis of previous work by us and others (9, 26), we hypothesized that systemically administered IT-901 may be efficacious in the treatment of acute GVHD, an immunologic disorder that critically depends on NF-κB and specifically c-Rel activity for T-cell activation by alloantigens (9). We chose a treatment dose of 24 mg/kg based on our PK studies and dose-response *in vitro* data, with the goal of reaching IT-901 serum levels within the expected therapeutic range (0.5–1 μg/mL, equivalent to 1.5–3 μmol/L). Initiation of i.p. IT-901 treatment within one week after HSCT was poorly tolerated and therefore not feasible. This phenomenon can likely be attributed to the low level of stress tolerance of mice in the immediate time period post lethal irradiation. Moreover, NF-κB inhibition in the setting of acute exposure to high-dose irradiation may sensitize tissues such as the intestinal epithelium to radiotoxicity (27). When initiating treatment after day 7 following HSCT, gut toxicity was still dose limiting, precluding daily inhibitor administrations (not shown). However, administration every other day starting on day 8 after HSCT was well tolerated and effectively improved the severity of lethal acute GVHD (Fig. 2A and Supplementary Fig. S6). Of note, IT-901 was again more efficacious than IT-603, and the therapeutic benefit was sustained even after cessation of c-Rel inhibitor treatment. Moreover, IT-901 treatment did not abolish the antitumor activity of donor T cells (Fig. 2B and C), reinforcing our previous observation that inhibition of c-Rel activity improves GVHD without impairing antitumor responses (9). Consistent with our previous results with IT-603, we demonstrated an increase of the percentage of donor regulatory T cells following IT-901 treatment during GVHD, but otherwise no differential effect on the immune status of the recipients (Fig. 2D and Supplementary Fig. S7).

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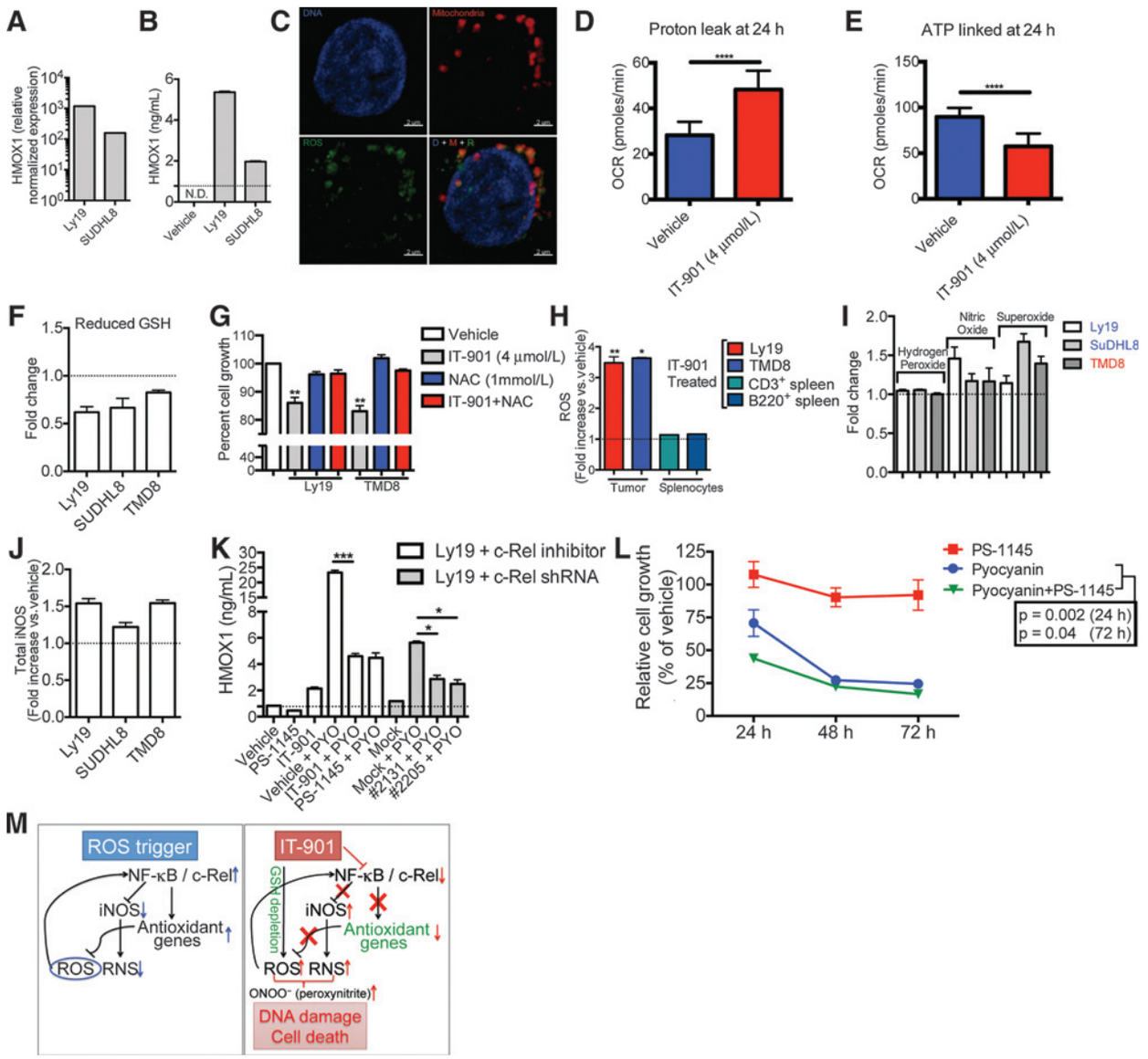

Figure 5.

Antilymphoma efficacy of IT-901 is mediated by oxidative stress. A and B, Ly19 and SU-DHL8 tumor cells were treated with IT-901 (4 μmol/L), or control vehicle for 24 hours. Heme oxygenase 1 (HMOX1) gene expression level by qPCR and protein level by ELISA are shown (A and B), respectively. Dotted line indicates the minimum detection limit of the assay. N.D, not detected. C, SU-DHL8 tumor cells were treated with IT-901 (4 μmol/L) for 24 hours and stained with carboxy-H_2DCFDA for microscopic detection of ROS. Representative confocal microscope images are shown (blue; DNA, red; Mitochondria, green; ROS; for more experimental details, see Materials and Methods). Data are representative of two independent experiments. D and E, Ly19 cells were treated with IT-901 at 4 μmol/L or control vehicle for 24 hours and mitochondrial respiration was analyzed with extracellular flux assays (see Materials and Methods for more experimental details). Proton leak (= non-ATP linked respiration; D) and ATP-linked OCR are presented (E). ****, $P < 0.0001$. F, Ly19, SU-DHL8, and TMD8 cells were treated *in vitro* with IT-901 (4 μmol/L) or control solution for 24 hours. Cells were analyzed for total and oxidized glutathione by HT Glutathione Assay Kit as described in Materials and Methods and the levels of reduced glutathione are presented. Values represent mean ± SEM. $P = 0.015$ when mean levels of reduced GSH from each cell line ($n = 3$) were compared with control. G, Ly19 and TMD8 tumor cells were treated with IT-901 (4 μmol/L) or control vehicle solution for 24 hours with or without *N*-Acetyl-L-cysteine (NAC). Relative cell growth was measured by MTS Cell Proliferation Assay. Values represent mean ± SEM ($n = 4$, technical replicates). **, $P < 0.01$. H, Ly19 and TMD8 tumor cells were treated with IT-901 (4 μmol/L) or control vehicle solution for 24 hours to analyze reactive oxygen species (ROS). Wild-type C57BL/6 mouse splenocytes were also cultured in the same condition. ROS levels were analyzed by utilizing a fluorogenic marker (carboxy-H_2DCFDA) and normalized to the level of vehicle-treated cells. Values represent mean ± SEM ($n = 4$, technical replicates). *, $P < 0.05$; **, $P < 0.01$. I, Ly19, SU-DHL8, and TMD8 tumor cells were treated with IT-901 (4 μmol/L), or control vehicle for 24 hours. Levels of hydrogen peroxide, nitric oxide, and superoxide after the treatment are shown (normalized values to vehicle treatment, dotted line indicates the level of control vehicle-treated group). Values represent mean ± SEM ($n = 3$, technical replicates). Data are representative of two independent experiments. J, Ly19, SU-DHL8, and TMD8 cells were treated *in vitro* with IT-901 (4 μmol/L) or control solution for 24 hours. Total iNOS levels are shown in as determined by cell-based human total iNOS immunoassay kit (ELISA). $P = 0.023$ when mean iNOS activities from each cell line ($n = 3$) were compared with control. (*Continued on the following page.*)

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IT-901 inhibits tumor growth in a xenograft model of human EBV-induced B-cell lymphoma

Rel/NF-κB proteins can act as oncogenic transcription factors by contributing to tumor growth, survival, drug resistance, and metastasis (1, 7). The combination of c-Rel inhibition and T-cell therapy may therefore not only be advantageous in that this immunomodulatory approach allows for the separation of GVHD from GVL activity, but inhibition of c-Rel activity may also display a direct antineoplastic effect. Recent genetic evidence has established a role for NF-κB signaling in lymphoid malignancies (28). To provide a rationale for clinical application of our c-Rel inhibitor technology to lymphoid malignancies, we analyzed c-Rel gene expression in various human cancer cells. We found particularly high expression of the c-Rel gene in human EBV-transformed B cells and diffuse large B-cell lymphoma (DLBCL) cell lines (Supplementary Fig. S8A and S8B). Moreover, we demonstrated nuclear translocation of c-Rel (indicating constitutive c-Rel activity) in a wide range of human primary lymphoma cells, especially in DLBCL and EBV-transformed B cells (Supplementary Fig. S8C). Treatment of a human EBV-B cell lymphoblastoid cell line (BLCL) with IT-901 *in vitro* induced inhibition of cell growth, whereas the numbers of normal B lymphocytes were not altered by IT-901 treatment (Fig. 3A). We next analyzed the *in vivo* efficacy of IT-901 in a human EBV-induced B-cell lymphoma xenograft model and found that treatment with IT-901 significantly inhibited tumor growth (Fig. 3B). In addition, the area of necrosis was significantly larger in tumors harvested from mice receiving IT-901 (Fig. 3C). EBV-associated lymphoma is a rare but life-threatening complication occurring mostly in severely immunocompromised patients in the setting of allogeneic HSCT (29). Immunotherapy with EBV-specific cytotoxic T lymphocytes has been shown to be an effective treatment option for this disease (30). Importantly, IT-901 treatment did not compromise the efficacy of T-cell therapy in this model (Fig. 3D and Supplementary Fig. S9), underscoring that in contrast with alloimmunity, protective antilymphoma immunity mediated by antigen-specific T cells is not impaired by IT-901 treatment (see ref. 9 for additional mechanistic information).

IT-901 inhibits growth of human DLBCL cells

On the basis of gene expression profiling, it is well known that there are two molecularly distinct forms of DLBCL: activated B-like (ABC) and germinal center B-like (GCB) DLBCL (31). We therefore analyzed nuclear translocation of c-Rel as well as of three additional NF-κB family members (p50, p52, and p65) in six representative DLBCL cell lines including ABC (HBL1, TMD8, U2932) and GCB (Ly19, SU-DHL4, SU-DHL8) cell lines and found that c-Rel and overall NF-κB activity was highest in ABC DLBCL cells (Supplementary Fig. S10). Importantly, IT-901 treatment strongly suppressed constitutive c-Rel activity of DLBCL cells but had a weaker effect on p50 and p52 activity and only mildly affected p65 activity (Fig. 4A). The "cross-reactivity" of IT-901 regarding Rel proteins other than c-Rel does not come as a surprise given that this family of proteins is structurally related (32). Although IT-901 treatment decreased cell viability in a dose-dependent fashion, at least 60 percent of cells were still viable after 48 hours of IT-901 treatment (4 μmol/L) in all tested cell lines except HBL1 (Fig. 4B and C).

We next investigated whether IT-901 treatment affected growth of DLBCL cells *in vitro*. To our surprise we found that IT-901 inhibited cell growth of both ABC and GCB cell lines with the IC_{50} values between 3μmol/L to 4μmol/L (Fig. 4D and Supplementary Fig. S11). In addition, IT-901 resulted in decreased proliferation of viable ABC and GCB DLBCL cells (determined by Ki-67 expression; Fig. 4E). This suggests that the decreased lymphoma cell numbers in response to IT-901 treatment were not just the result of cell death but also due to slower cell growth.

Inhibition of c-Rel identifies TNFα as a possible therapeutic target in ABC DLBCL cells

NF-κB plays an important role in the initiation and promotion of cancer by fostering an inflammatory milieu in which various cytokines aid and abet malignant transformation (33). To examine whether IT-901 alters cytokine production by DLBCL cells, we analyzed cytokine levels in the supernatant after *in vitro* incubation with IT-901. As anticipated, cytokine levels were hardly detectable in GCB DLBCL cells (not shown), in contrast with ABC DLBCL cells, where TMD8 showed marked production of various cytokines. IT-901 treatment resulted in decreases of most cytokine levels in TMD8 cells, with the notable exceptions of IL8, TNFα, and TNFβ (Fig. 4F). Interestingly, TMD8 was the only DLBCL cell line that did not show inhibited growth when cultured with IT-901 at a concentration of 3 μmol/L (Fig. 4D), suggesting that in TMD8 cells cytokines such as IL8 and TNFα may be increased as a mechanism to overcome c-Rel inhibition by compensatory upregulation of NF-κB–dependent survival pathways. Indeed, *in vitro* treatment of TMD8 cells with a TNFα neutralizing antibody inhibited cell growth, and this effect was enhanced when combining TNFα blockade with IT-901 treatment (Fig. 4G), suggesting that the TNFα pathway may be a therapeutically exploitable target in some B-cell lymphomas.

(*Continued*.) K, Ly19 tumor cells were treated with pyocyanin (PYO) to generate high levels of oxidative stress in the presence of vehicle versus IT-901 or PS-1145 (at 4 μmol/L, open bars), or in the presence of mock versus c-Rel-specific shRNA (#2131 and #2205, gray bars) for 24 hours, followed by analysis of HMOX1 induction. HMOX1 protein level by ELISA is shown. Dotted line indicates the minimum detection limit of the assay. Values represent mean ± SEM ($n = 5$, technical replicates). *, $P < 0.05$; ***, $P < 0.001$. See Materials and Methods for more experimental details. L, Ly19 cells were treated for 3 days with PS-1145 (4 μmol/L), pyocyanin (350 μmol/L), or PS-1145 (4 μmol/L) + pyocyanin (350 μmol/L). Relative cell growth was measured by MTS assay. Combined data from two independent experiments are presented. M, the proposed effects of IT-901 on oxidative stress induction and the oxidative stress response in lymphoma cells. Left, upon ROS induction, HMOX1 is upregulated through NF-κB activation and this serves as an antioxidant response. At the same time, the level of iNOS is expected to be decreased as iNOS production is negatively regulated by the NF-κB pathway. Impacts of ROS induction are indicated by blue arrows. Right, in the setting of IT-901 treatment, three major responses are observed: (i) buildup of ROS as a result of GSH depletion; (ii) suppression of HMOX1 due to decreased NF-κB/c-Rel activity, which weakens the oxidative stress response; and (iii) induction of iNOS by inhibiting its negative regulator NF-κB/c-Rel, resulting in production of nitric oxide. Increased production of ROS/RNS contributes to the production of peroxynitrite, which exacerbates ROS-mediated DNA damage and cell death. Impacts of IT-901 are indicated by red arrows and cross marks. Changes that affect ROS removal are shown in green.

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IT-901 mediates anti-lymphoma activity by selective induction of oxidative stress in lymphoma cells

The nonselective inhibitory effect of IT-901 on growth of both ABC and GCB DLBCL cells led us to conclude that an NF-κB–independent mechanism contributed to the antilymphoma activity of IT-901. Various interactions between the oxidative stress response and NF-κB signaling have been proposed (34). Oxidative stress is an important element of anticancer therapies, although it has been implicated in tumorigenesis as well (35, 36). Heme oxygenase-1 (HMOX1) is an oxidative stress response gene (37) whose expression is regulated by NF-κB. We found that HMOX1 gene expression was highly upregulated in DLBCL cells treated with IT-901 for 6 hours (Fig. 5A). Furthermore, we detected significantly increased HMOX1 protein levels in Ly19 and SU-DHL8 cells treated with IT-901 for 24 hours (Fig. 5B) suggesting that HMOX1 expression was induced, which is a hallmark of oxidative stress. Oxidative stress is mediated by ROS that are created by a variety of cellular processes including electron leakage from mitochondrial respiratory complexes (38). We therefore next checked for the presence of ROS in cells treated with IT-901 and found that, as expected, ROS production was largely localized within mitochondria (Fig. 5C and data not shown). Interestingly, analysis of mitochondrial respiration in Ly19 cells that were incubated with IT-901 for 24 hours revealed increased mitochondrial proton leak (equivalent with non-ATP linked oxygen consumption; Fig. 5D). It is a well-known phenomenon that there is a positive feedback loop between ROS and proton leak, reflecting cellular adaption to oxidative stress (39). Consistent with increased proton leak, ATP-linked mitochondrial respiration was decreased in IT-901–treated cells, indicating mitochondrial dysfunction (Fig. 5E).

Oxidative stress is the result of an imbalance between generation and elimination of ROS. One of the major ROS-scavenging systems is glutathione (GSH; ref. 40). Given that the IT-901 molecule has redox properties, we hypothesized that IT-901 may alter the GSH homeostasis in affected cells. Indeed, we found that the levels of reduced GSH of DLBCL cells were significantly decreased following IT-901 treatment (Fig. 5F), suggesting that depletion of the reduced GSH pool by IT-901 initiates the increase in ROS levels. Replenishing the GSH pool of DLBCL cells by the addition of the antioxidant *N*-Acetyl-L-cysteine completely reversed the inhibitory effect of IT-901 on DLBCL growth, indicating that induction of oxidative stress by GSH modulation was indeed the main factor contributing to the antilymphoma activity of IT-901 (Fig. 5G). IT-901 induced production of high levels of ROS in both ABC and GCB DLBCL cells (Fig. 5H). Of note, normal leukocytes generated no ROS in response to treatment with IT-901 (Fig. 5H and Supplementary Table S2), suggesting that lymphoma cells are particularly sensitive to induction of oxidative stress by IT-901. Analysis of several reactive oxygen and nitrogen species revealed that superoxide and nitric oxide (NO) but not hydrogen peroxide levels were increased in lymphoma cells treated with IT-901 (Fig. 5I). Of note, superoxide and NO react spontaneously to form the highly toxic oxidant peroxynitrite, which is known to cause lipid, protein, and DNA damage, and therefore cell death (34). NO can be produced by various cell types that are capable of expressing inducible NO synthase (iNOS). iNOS is an NF-κB target gene and it has been previously shown that NF-κB activation in Burkitt lymphoma cells was associated with inhibition of NO production (41). Consistent with this report we found that treatment with the NF-κB inhibitor IT-901 increases iNOS activity in B-cell lymphoma cells (Fig. 5J).

Finally, we sought to investigate the impact of c-Rel inhibition on the oxidative stress response. We hypothesized that IT-901 would not only induce oxidative stress, but also suppress antioxidant genes via inhibition of NF-κB target genes involved in the oxidative stress response such as HMOX1. We treated Ly19 cells with the pro-oxidant pyocyanin to generate high levels of oxidative stress, followed by analysis of HMOX1 (using HMOX1 as a surrogate marker of the NF-κB–dependent oxidative stress response) in the presence of vehicle versus NF-κB inhibitors (IT-901 or the IKK inhibitor PS-1145; ref. 42; Fig. 5K, open bars), or in the presence of mock versus c-Rel–specific shRNA (Fig. 5K, gray bars). We found that c-Rel/NF-κB inhibition with PS-1145, IT-901, or shRNA indeed modulated the oxidative stress response, exemplified by reduced HMOX1 induction in the presence of pyocyanin. We therefore next investigated whether NF-κB inhibition on the setting of oxidative stress had the potential to sensitize lymphoma cells to oxidative stress-mediated cell damage. We cultured Ly19 cells in the presence of oxidative stress without NF-κB inhibition (pyocyanin alone), in the presence of NF-κB inhibition in the absence of oxidative stress (PS-1145 alone), or in the presence of oxidative stress in the setting of NF-κB inhibition (pyocyanin + PS-1145, which is equivalent to the proposed dual mechanism of IT-901; Fig. 5L). We found that NF-κB inhibition alone had no effect on Ly19 growth, induction of oxidative stress strongly inhibited lymphoma cell growth, and combining oxidative stress and NF-κB inhibition had additive antilymphoma effects. The proposed effects of IT-901 on oxidative stress induction and the oxidative stress response are summarized in Fig. 5M.

Discussion

Although the NF-κB pathway is involved in a multitude of physiologic and pathophysiological processes affecting both hematopoietic and nonhematopoietic cells, healthy cellular steady state is not associated with NF-κB activity. A truly NF-κB–specific drug would therefore not be expected to disturb normal cell function. Clinical strategies resulting in suppression of NF-κB activity include proteasome inhibitors (43), Bruton's tyronsine kinase inhibitors (44, 45), thalidomide and its analogs (46, 47), amongst others. None of these approaches is strictly NF-κB–specific, enhancing the potential of these agents for both therapeutic and adverse effects. Increasing NF-κB specificity of a given drug would be expected to improve the safety profile, possibly at the expense of reducing efficacy for some indications (such as cancer). Here, we show that small-molecule–mediated inhibition of the NF-κB pathway at the transcription factor level can efficiently be accomplished *in vivo* and is associated with little toxicity. Moreover, we are able to demonstrate improved *in vivo* efficacy of a multidose c-Rel inhibitor treatment regimen for acute lethal GVHD. The importance of this finding should not be underestimated given the issues with proteasome and IκB kinase (IKK) inhibitors in exacerbating acute GVHD (48, 49). Furthermore, our data confirm our previous findings (9) that both antitumor and anti-viral activity of T cells is not compromised by c-Rel inhibitor treatment, adding to the favorable safety profile.

c-Rel is well known for its role as an oncogenic transcription factor, contributing to constitutive NF-κB activity in a wide range of cancers (7), most notably in lymphoid malignancies (50–52). We show that c-Rel is particularly overexpressed and constitutively

active in DLBCL cells and EBV-transformed B lymphoblasts. As expected, *in vitro* c-Rel inhibitor treatment modulated the cytokine profile of DLBCL cells with high NF-κB activity (ABC but not GCB DLBCL cells), consistent with a previous study describing differential effects of IKK inhibitor treatment on ABC versus GCB DLBCL cells (42). In contrast with this, IT-901 had an inhibitory effect on cell growth of both of these distinct DLBCL types, suggesting that NF-κB inhibition was not the main factor affecting lymphoma cell behavior in the presence of the small molecule. We identified an additional mechanism of action of IT-901 that takes advantage of the fact that ROS production is increased in cancer cells and that modulation of the redox homeostasis has potential to be therapeutically exploited as a method to selectively cause toxicity to cancer cells while sparing healthy tissues (40). Importantly, there is a threshold range of ROS-mediated effects on cellular function. ROS levels below the critical threshold may actually be beneficial by stimulating pro-survival signaling pathways, whereas levels exceeding the threshold become harmful and result in cell death (40, 53). We found that culturing cells in the presence of IT-901 depletes their reduced GSH pool, which impairs ROS elimination and results in superoxide accumulation in B-cell lymphoma cells (but not in normal leukocytes where basal superoxide generation is negligible; ref. 54). IT-901–mediated NF-κB inhibition further exacerbated oxidative stress in lymphoma cells by inducing iNOS activity (thereby generating nitric oxide and enabling formation of peroxynitrite) while inhibiting oxidative stress response elements that are regulated by NF-κB (as illustrated by modulation of a representative oxidative stress response gene; HMOX1). Importantly, we were able to demonstrate additive antilymphoma effects of combination treatment with a pro-oxidant in the presence of an NF-κB inhibitor. IT-901 therefore represents a novel small molecule acting as a direct c-Rel/NF-κB inhibitor with additional antineoplastic activity secondary to selective induction of oxidative stress in lymphoma cells (the latter mechanism being exacerbated by NF-κB inhibition).

Interestingly, exposing lymphoma cells to IT-901 in the presence of an antioxidant reversed the anti-lymphoma effect of the small molecule even in ABC DLBCL cell lines depending on constitutive NF-κB activity for their survival. These observations reinforce the notion that for the treatment of malignant diseases, small-molecule–mediated NF-κB inhibition at the transcription factor level may not be sufficient to mediate strong anti-neoplastic activity in a single-agent treatment regimen. However, consistent with NF-κB inhibition, IT-901 modulated the cytokine profile of ABC DLBCL cells, leading us to identify TNFα as a potential therapeutic target in activated B-cell lymphomas. Combination of c-Rel/NF-κB inhibition with additional cytostatic or targeted approaches has significant potential for the development of innovative minimally toxic cancer treatment protocols. Examples for rational combination strategies include NF-κB inhibition + inhibition of upstream targets such as TNFα (Fig. 4G), tyrosine kinases, VEGF, or c-Rel inhibition + checkpoint blockade such as cytotoxic T lymphocyte antigen 4 (CTLA4) inhibition (where c-Rel inhibition could mitigate adverse effects related to increased autoreactivity in the setting of reduced peripheral tolerance).

Taken together, we introduce the novel small-molecule IT-901, a compound with unique properties that may benefit allogeneic transplantation as well as cancers therapy. Because of its strong c-Rel/NF-κB inhibitory potency combined with its capacity to induce high levels of oxidative stress specifically in lymphoma cells, the overall efficacy of IT-901 is far superior to our previous drug candidate IT-603. IT-901 has significant potential as antineoplastic agent for the treatment of hematologic malignancies, especially in the setting of allogeneic HSCT where c-Rel inhibition has the added benefit of ameliorating GVHD. Moreover, our findings indicate that IT-901 is not only efficacious and well tolerated but our PK and toxicology data suggest that this molecule is a promising candidate for clinical drug development. Issues that will need to be addressed before application to human subjects include additional investigational new drug–directed toxicology studies, as well as the development of an optimized clinically acceptable formulation. We expect to be able to launch a first-in-man clinical trial within the next 3 years.

Disclosure of Potential Conflicts of Interest

H.-C. Liou has ownership interest (including patents) in ImmuneTarget. M.R.M. van den Brink is a consultant/advisory board member for Novartis. J.L. Zakrzewski is a consultant/advisory board member for ImmuneTarget Inc. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

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Authors' Contributions

Conception and design: Y. Shono, H.-C. Liou, E. Doubrovina, E. Derenzini, S. Ouk, J.J Tsai, A. Younes, M.R.M. van den Brink, J.L. Zakrzewski

Development of methodology: Y. Shono, A.Z. Tuckett, E. Doubrovina, M. Doubrovin, J.L. Zakrzewski

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Shono, A.Z. Tuckett, E. Doubrovina, E. Derenzini, O.M. Smith, E.R. Levy, F.M. Kreines, M.I. Scallion, M. Doubrovin, R.J O'Reilly, M.R.M. van den Brink, J.L. Zakrzewski

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Shono, A.Z. Tuckett, E. Doubrovina, E. Derenzini, S. Ouk, C.G.K. Ziegler, M. Doubrovin, G. Heller, A. Younes, M.R.M. van den Brink, J.L. Zakrzewski

Writing, review, and/or revision of the manuscript: Y. Shono, A.Z. Tuckett, E. Doubrovina, E. Derenzini, M. Doubrovin, R.J O'Reilly, M.R.M. van den Brink, J.L. Zakrzewski

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Doubrovina, E.R. Levy, F.M. Kreines, M.I. Scallion, A. Younes, R.J O'Reilly, M.R.M. van den Brink, J.L. Zakrzewski

Study supervision: M.R.M. van den Brink, J.L. Zakrzewski

Other (provided cell products for the studies): E. Doubrovina

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